Claim 53 (new). 53. The method of claim 52, wherein said step of performing an analysis includes conducting a melting point analysis to identify said first and second amplification products and to evaluate whether their respective structures include mutations.

Claim 54 (new). 54. The method of claim 53, including the further step of measuring the intensity of the detected signal during the melting analysis in order to evaluate the relative quantity of each amplification product melted as a function of the intensity of its signal.

Remarks/Arguments

The Applicant requests the entry of the amendments to the specification and claims as proposed above. The amendments place the claims in a form for allowance and otherwise comply with 37 C.F.R § 1.116.

In the specification, the amendments to the paragraphs noted are minor editorial revisions with no introduction of new matter.

Claims 1-16, and 18-19, have been cancelled. Claim 17 has been amended. Claims 20 - 54 have been added and are new claims. What follows is a response to the Examiner's claim rejections.

Claim Rejection – 35 U.S.C. § 112

The claims have been amended to eliminate the rejection based on the word "forming" used in the claims. The claims now refer to "obtaining" a nucleic acid sample, and

"synthesizing" forward and reverse primers. As described on pages 20-21 of the Application, the method involves obtaining a nucleic acid sample (in the example given, mRNA) having a selected target sequence, and then synthesizing forward and reverse primers that are structured so that they will hybridize to opposite complementary strands at pre-determined locations proximate the selected target sequence found in the nucleic acid. As noted in pages 13-14, and page 20, the target sequences on nucleic acid are selected, and forward and reverse primers are then designed and synthesized in coordination with the target sequences. The forward and reverse primers of the primer pair are also synthesized to contain specific fluorescent dyes, that only interact and produce a fluorescent signal when they are positioned in close proximity, within a specific distance. In the present method, when the forward and reverse primers interact to produce a fluorescent signal, this indicates they have each become integrated into the opposite complementary strands of a double-stranded amplification product, in the pre-determined locations proximate the selected target sequence, which places them within the specific distance that allows the fluorescent moieties of the two primers of the pair to interact and produce a signal.

In addition and as noted in the Application, p.22, the term "measuring device" refers to a fluorimetric device that detects specific fluorescent signals generated by the interaction of fluorescent moieties of the primer pairs of the invention, as well as fluorescent signal intensity, including changes in intensity. In the present Application, the term "fluorimetric" as opposed to

"spectrophotometric" as suggested by the Examiner, is a more accurate descriptor of the measuring device. An example of the measuring device referenced in the claims is the Roche Light Cycler™, as described on p.22, a multi-channel fluorimeter with thermocycling capabilities. The Application also describes an example of the measuring device as a spectrofluorimeter, at page 12. Electrophoresis may be used in control experiments to determine amplification reaction products (see page 23), but is not used to detect fluorescent signals or signal intensity. As noted in the attached claim amendments, the claims have been amended to reference a fluorimetric device.

Claim Rejection – 35 U.S.C. § 102

Claims 1-5 and 6-15 are rejected under 35 U.S.C. § 102(e) pursuant to Nazarenko, U.S. Patent No. 6,090,552.

Nazarenko principally teaches the use of hairpin primers (see Col. 6, lines 58-64, where the use of hairpin primers is described as a preferred embodiment) , which have a completely different structure and function than the paired forward and reverse primers of the present method. In virtually all of the applications described in Nazarenko, the fluorescent signal indicating progress of an amplification reaction is produced by the interaction of two fluorescent moieties located within the same hairpin primer. When unreacted hairpin primer is in solution, the hairpin primer structure is closed, such that the fluorescent moieties of the hairpin primer are positioned proximate one another in the closed loop structure of the hairpin. One moiety is a

"quencher" fluorophore that serves to quench the fluorescent signal of the second fluorescent moiety found in the hairpin primer, the donor fluorophore. When the hairpin primer opens or unfolds as it becomes incorporated in a single nucleic acid strand, or is subject to the action of an enzyme such as DNA polymerase, the fluorescent moieties of the hairpin primer become separated, such that the quenching moiety's effect is attenuated, permitting the emitting donor fluorescent moiety to produce a detectable signal. Good examples of this are the reactions described in Figs. 3 and 5 of Nazarenko. The reaction of Fig. 3, discussed at Col. 26, lines 29-46, involves individual hairpin primers that become incorporated on nucleic acid strands and only produce a signal when DNA polymerase acts upon and disrupts the hairpin primer causing the quencher and donor fluorophore of each primer to become separated. The signal is produced by an individual hairpin primer whose donor fluorophore has become separated from the quencher of the same primer. The signal is not produced by the interaction of fluorescent moieties on two primers that are structured to be integrated in close proximity on opposite complementary strands so that the fluorescent moieties can interact to produce a signal. The reaction described by Fig. 5 involves the same effect, of a hairpin primer that is caused to unfold by the reaction to produce a fluorescent signal: "the hairpin unfolds, the quencher and fluorophore are separated, and a fluorescent signal is emitted from the amplification product," Col. 27, 17-20. Nazarenko fundamentally does not describe the synthesis of coordinated fluorescently tagged linear primer pairs that are structured to integrate into a double stranded amplification product in specific locations during polymerase chain reaction and that produce a

fluorescent signal by interaction between the two primers of the primer pair when they have achieved their pre-determined location in close proximity on the opposite complementary strands.

The Office Action references Nazarenko, Col. 29, lines 51-61, which involves the use of hairpin primers in strand displacement amplification (SDA). In this application, Nazarenko describes the use of forward and reverse hairpin primers, in which the fluorescent moiety of the forward primer does not interact with the fluorescent moiety of the reverse primer. Rather, and as described in Step 2, Fig. 10, and Col. 29, line 64, through Col. 30, line 5, each of the forward and reverse hairpin primers reacts in typical fashion, with a fluorescent signal being generated when the hairpin primer is incorporated and extended causing the hairpin to open, separating the quencher moiety of the hairpin primer from the donor fluorophore of the same hairpin primer, allowing the donor fluorophore to produce a fluorescent signal. There is no fluorescent interaction between the forward and reverse primers. There is no coordination of the location of the forward and reverse primers on opposite complementary strands of a double stranded amplification product flanking a target sequence. As an additional distinction, strand displacement amplification is a very different methodology from polymerase chain reaction, and the present method is stated within the context of polymerase chain reaction.

The Office Action also references several other sections of the Nazarenko specification, Col. 14, lines 29-53; Col. 13, lines 26-43; Col. 18, lines 22-35; Col. 49, lines 42-51; Col. 41,

lines 12-16; and Col. 51, lines 59-67, that are very broad descriptions of elements of amplification reactions and techniques that may be used in such reactions, but none of these references contains any description of the specific method of the present invention. For example, Col. 14, lines 29-53, does not contain any specifics of a methodology to be used. It speaks generally of using primers so that one primer has both a donor moiety and an acceptor moiety of a molecular energy transfer pair (as do hairpin primers), and that a second primer has a different donor moiety, and presumably a different acceptor moiety. In other words, it is clear that Col. 14, lines 29-53 describes a system of primers wherein each primer produces fluorescent signals independently—this reference is completely lacking any description of primers that interact with each other to produce a signal. Further, there is no discussion at all of the location of the primers when they are incorporated into an amplification product, i.e., whether they are on opposite complementary strands, and whether they are positioned in close proximity to allow for interaction between donor and acceptor fluorophores on the two primers of the primer pair. The references to Columns 18, 49, 41 and 51 similarly describe general methodology that is used in the field, but no specific description of the steps of the present invention.

The reference to Column 13 also is a very general introduction, but contains no specific steps of a methodology like that of the present invention. It generally references triamplification, which, it should be noted, is a very different process from polymerase chain reaction of the present method. In triamplification, as described in Nazarenko, Section 5.2.2 and 5.4.2, forward

and reverse primers bind at distant locations on opposite complementary strands, but do not interact to produce a fluorescent signal. Only one of the forward and reverse primers is fluorescently labeled, such that they cannot interact to produce a fluorescent signal, as in the present invention. A blocking oligonucleotide with a fluorescently flagged probe (not a primer) binds to a nucleic acid strand in a position partially complementary to a primer on the opposite strand. The blocking oligonucleotide does not (in fact, can not) operate as a primer, but functions to prevent the extension of a new nucleic acid strand from the forward primer, and must be ligated to the nascent nucleic acid strand extending from the primer with a separate enzyme not used in polymerase chain reaction, such as DNA ligase. Further, the blocking oligonucletide essentially overlaps the primer on the opposite strand, as opposed to being positioned in a location flanking a target sequence. The result is that the unreacted blocking oligonucleotide and reverse primer in solution have a tendency to bind to each other in the reaction mixture, producing false positive signals. This is what necessitates exonuclease treatment mentioned in the Nazarenko description, not found in amplification by polymerase chain reaction, to disable the fluorescent tag on the probe. Triamplification is not comparable to polymerase chain reaction, and involves additional reaction steps, enzymes (such as DNA ligase), and reactants (such as blocking oligonucleotides). Further, and fundamentally, triamplification does not include the use of forward and reverse primers fluorescently tagged so that they interact when used to build and become incorporated into opposite complementary nucleic acid strands in polymerase chain reaction as described in the present Application.

Page 4 of the Office Action references Col. 24, lines 57-64 for the proposition that Nazarenko describes the positioning of fluorescent moieties separated by 15-25 nucleotides or about 100 Angstroms. This section of Nazarenko actually emphasizes the differences between Nazarenko's methodology and the present Applicant's invention. Column 24, lines 57-64 describes the structure of a hairpin primer, where the two fluorescent moieties are both located on the nucleotide loop of the same hairpin primer. When the primer is in the closed configuration, the fluorescent moieties are located close to each other on opposite sides of the closed nucleotide loop of the same primer, at a distance of about 1 nucleotide, such that the "quencher" moiety quenches the signal of the donor fluorophore. See Col. 24, lines 50-56 and Fig. 4. When the loop of the hairpin primer is open and extended, the quencher is located about 20 nucleotides away from the donor fluorophore, a large enough distance that the quencher no longer can quench the signal emitted by the donor fluorophore. To produce a signal, the fluorescent moieties of a hairpin primer must be separated and positioned apart, not brought close together.

In the method of the present Application, the interacting fluorescent moieties are on two primers, not the same primer. Also, the methodology of the present Application involves utilizing moieties on the two primers of a primer pair that produce a signal, or some other fluorescent effect, when the primers and their moieties are brought close together, the opposite of what is described in Nazarenko. The method involves bringing the forward and reverse primers

and their fluorescent moieties in close proximity, in a preferred embodiment within a distance of about 100 Angstroms, and when they are brought within that distance, either a fluorescent signal is produced, or some other fluorescent event occurs, which can be detected by a fluorimetric measuring device. In fact, the fluorescent moieties used with hairpin primers, a quencher and a donor fluorphore, can be used in the method of the present Application, except that they will be positioned on two separate primers (not the same primer), and the methodology will look for a reduction (as a result of quenching by the quencher moiety) in the emitting fluorophore's signal when the two moieties are brought close together as they are incorporated in close proximity flanking a relatively short target sequence.

The Office Action also references the use of Nazarenko's hairpin primers to identify mutations at Col. 56, lines 10-35 and Col. 57, lines 12-44, and to run multiplex reactions in the context of polymerase chain reaction, Col. 49, lines 12-19. The present Application does not claim the broad concept of identifying mutations or of running multiplex reactions, and neither apparently does Nazarenko, but rather claims the new methodology of the present Application as applied to identifying such mutations and running multiplex reactions. The fact that Nazarenko has addressed the same problems with a different method does not justify rejection of claims applying the present, new method to these problems.

The Office Action also references Col. 49, lines 1-6 of Nazarenko for the determination of the length of the target sequence. The electrophoresis method described in Nazarenko is

merely a validation methodology, however, and has to do with verifying that the PCR products are consistent with expectations and the fluorescent findings. Nazarenko was not using electrophoresis as a diagnostic method, and he does not appear to use real time fluorescent methods to measure the varying length of the PCR products (amplicons) by evaluating the distance between fluorophores to determine lengths of amplicons and target sequences. The method of the present Application does not employ electrophoresis to analyze amplification products; rather, it employs the real time analysis of the fluorescent signal(s) generated by the fluorescent moieties on primers incorporated in amplification products, as a way of analyzing the length and varying lengths of the original target sequences. The intensity of the signal can be used to analyze the distance separating the fluorescent moieties in the amplification products. The reference to electrophoresis is not relevant to the present Application, although electrophoresis can be used as a validation methodology with the present method as it has apparently been used in Nazarenk.

The Office Action also references the use by Nazarenko of a control experiment in the context of Nazarenko's universal hairpin primer application, at Col. 51, lines 38-39. The present method does not claim the use of a control, per se, but the use of a control within the context of the new method of the present Application. The fact that Nazarenk may have used a control with a different hairpin primer method does not justify rejection of the present claims.

To conclude, as to the Section 102 rejection, Nazarenko describes a fundamentally different method and applications, and plainly does not teach the present method.

Claim Rejection- 35 U.S.C.§ 103.

As to the Section 103 rejection, the Office Action repeats the references to Nazarenko cited in the portion of the Office Action concerning the Section 102 rejection. Those references have been discussed above, and Nazarenko plainly does not teach or even suggest the elements of the present method, including the use of forward and reverse primers that are fluorescently labeled and that interact to produce a fluorescent signal or other fluorescent event when brought in close proximity as they are incorporated into the opposite complementary strands of a double-stranded amplification product of polymerase chain reaction. The Office Action recognizes that Nazarenko does not teach the selection of a target sequence comprising a length of up to 130 nucleotides and a range from about 25 nucleotides to about 100 nucleotides, as well as mRNA and the use of the forward primer to produce a cDNA strand. The Office Action cites an article by Herrewegh and contends that Herrewegh teaches a method for reverse transcriptase PCR that involves using target nucleic acid sequence in the range of 35-177 bp and preparing RNA and reverse transcribing RNA with a premix comprising a primer used for PCR to produce cDNA.

Yet, it is clear from reading Herrewegh, and especially the description including Fig. 1, p. 685 and Table 2, p. 685, that there were two RNA target sequences amplified, one of the length of 233 nt and the other having the length 177 nt. The 35 bp amplification product referenced by

the Examiner was the result of Dra 1 restrictase DIGESTION of the 177 bp amplification product, as explained in Fig. 2 and the legend thereof, p. 686. In other words, Herrewegh does not describe the amplification of a 35 bp short target sequence, i.e. or any other target sequence in the range of about 25-100 nt. The picture at Fig. 2 is a good illustration of why very short amplifications have been historically taboo in the PCR paradigm—it is very challenging technically to visualize short fragments of DNA using the gel electrphoresis technique. The 35 bp product on Fig. 2 of Herrewegh can barely be seen and it is hard to determine its length, since the shortest marker in the reference lane has a length of 75 bp. As noted, this product was not the result of amplification, but the result of digestion, and it cannot be cited in support of the Section 103 rejection. Further, Herrewegh does not describe the forward and reverse primer method of the present Application as applied to reverse transcriptase PCR.

As a general note, ultra-short PCR products (35-135 bp in length) were traditionally avoided from the inception of PCR in 1985, because there was no analytical technique (or at least no simple analytical technique) to identify or quantify these short products. For over 10 years the predominant method for analysis of the results of PCR was electrophoresis, i.e. the method based on sizing of DNA amplicons. Sizing of very short DNA products when applying traditional electrophoretic methods can be challenging, because of the resolution limitations of the methods proper, and difficulties with detecting such short products. Practically, the only high quality option in this case was radioactive tags, which creates safety hazards and poses certain technical

problems (time consuming, laborious, specially trained personnel, etc.). It was also challenging to distinguish between specific and non-specific (primer-dimer) amplification products, since they were of similar sizes.

Also, the primary goal of early day PCR experiments was to isolate (or isolate-modify) nucleic acid sequences of interest in appropriate quantities with the purpose of cloning them in vectors and, subsequently, examine these sequences/function in the context of cellular (bacterial or eukaryotic) experimentation. That is why considerable efforts were put into amplifying whole genes, or substantial portion of the genes of interest, which can be in the region of tens of thousands of bases. Lately, however, the emphasis shifted to molecular diagnostic applications: i.e. identifying specific mutations (either single nucleotide polymorphisms or break points, or short insertions/deletions) and on quantifying expression levels of specific genes on RNA level, or identifying and quantifying foreign sequences (for example, viral or bacterial on the background of human). All of these tasks do not require "long PCR" products, quite opposite, they require high throughput, fast methods with elevated confidence of quantitative results with maximal sensitivity

The present method reverses the picture. The real-time fluorescence- and primer-based detection system of the Application performs best with shorter nucleic acid target sequences because short targets have, generally speaking a simpler structure, thus avoiding possible complications of primer interaction with nucleic acid regions of complex structure. The present

Application places primers in very close, adjacent (non-overlapping) proximity. This design produces substantial experimental advantages and is believed to be the first implementation of these principles.

Accordingly, the Applicant respectfully submits that the Application and claims as amended are in a form for allowance.

Applicant is enclosing a check in the amount of $465.00, to cover the three month extension of time and a second check in the amount of $168.00 to cover the additional claims fee. The Commissioner is hereby authorized to charge any additional fees to the Holme Roberts & Owen, LLP deposit account no. 08-2665.

Respectfully Submitted,
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